Expression of cellulases in plants...

Rationale: For an initial set of constructs, fuse E2 and E3 genes of *Thermomospora fusca* to MAC promoter and NOS terminator in pCGN1578 constructs similar to Dennis-321 (MnP) or 263 (LiP) but lacking any signal sequence. Based on published information, neither protein is heavily glycosylated (E3 is functional without glycosylation), so cytosolic localization should be compatible with function.

9/18/95

streaked from agar slants to LBAmp plates
D644 gave only 3 colonies (DH5α + pSZ10)
D651 " many " (" pSZ15)

patch 2 of each for mini-preps.

1/19/96 - prep. 1/4 plate each prep using "Vince Schulz" protocol ($(NH_4)OAc$ ppt., CTAB ppt.)
- resuspend in 50μl, load 2μl

From maps...

E2 - 348-1578

SmaI	2 sites
SalI	
NarI	2 sites
EcoRI	
SphI	
SacII	
Bst+EII	2 sites
BspMI	
PvuII	

E3 - 689-2363

KpnI	
BspMI	"
Bst+EII	"
SalI	"
NarI	"
SacII	
NcoI	
BstXI	
SacI	"
HpaI	
SmaI	

Thomas Ziegelhoffer [redacted]

EXHIBIT B

PENGAD-Bayonne, N.J.

E3 construct - PCR amplification

Overall strategy:

1) generate XbaI @ 5' end, EcoRI @ 3' end (PCR)
2) subclone into pUC19 as XbaI - EcoRI fragment
3) replace ~1.4kb KpnI - SmaI of resulting pUC-E3 clone with KpnI - SmaI of pJZ10 (from strain D(44) → sequence using universal primers)
4) clone XbaI - EcoRI fragment of fully w.t. pUC-E3 into 253-1 vector (3 way ligation - XbaI - EcoRI - HindIII)
5) clone expression cassette (MAC-E3-MAS tet) into pCGN1578 as BamHI - HindIII fragment

Custom primers from UWBC (see data sheets)

XbaE3 (DSN # 5500) - designed to create an XbaI site and ATG initiation codon at the normal leader cleavage site, generating the sequence NH_3-MET-ALA-GLY-Cys-Ser-...
25mer of ~16225 MW, resusp. to give 210 μM solution (~~348~~ μg in 100 μl)

RIE3 (DSN # 5499) - designed to create an EcoRI site immediately after the stop codon of E3.
23mer of ~14827 MW, resusp to give 205 μM solution (307 μg in 100 μl)

set up 1st 6 of buffer optimization set

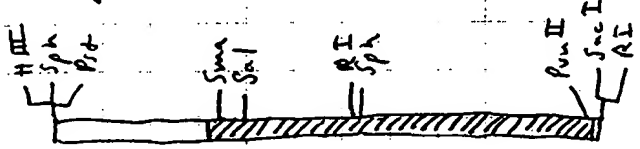
			combine
10 μl	10x buffer (#s 1-6)		
10 μl	4 dNTPs (2.5mM each)		
1 μl	template (1/1000 dilution of pJZ10 prep)		60
1 μl	10 μM	XbaE3	6
1 μl	10 μM	RIE3	6
~~77~~ μl	dH_2O		6
0.25 μl	Taq pol		~~480~~ μl dH_2O

(40 μl each rx)

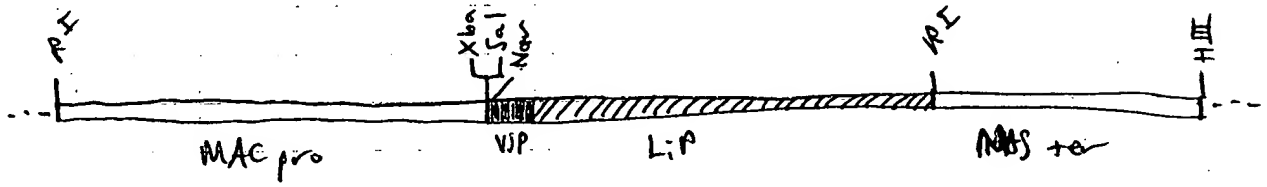
Method 1 change prog 31 to 48°C annealing temp. - Thomas

E2 cloning – strategy

E2 gene (in pUC19)

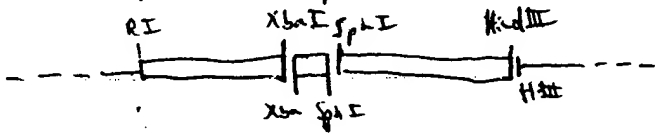


253-1 expression cassette (in pUC118, I think...)



Advantage: RI site available immediately downstream of 3' end of E2;
Problem: Need to avoid using EcoRI (2 sites in 253-1, 1 site in E2 gene)

final step in expr. cass. construction:



- vector backbone is 253-1, cut with XbaI & HindIII
- Xba-Sph fragment (~375 bp) is PCR-derived and contains the N-terminal end of E2 with new N-terminal Met (Met-Asn-Asp-Ser.... etc) This fragment will also be subcloned into pUC19 for sequencing to verify absence of errors
- Sph-Hind fragment (1.6kb) is derived from intermediate clone (~1.0kb RI fragment of E2 cloned into RI site of 253-1 [~2.5kb RI fragment excised])

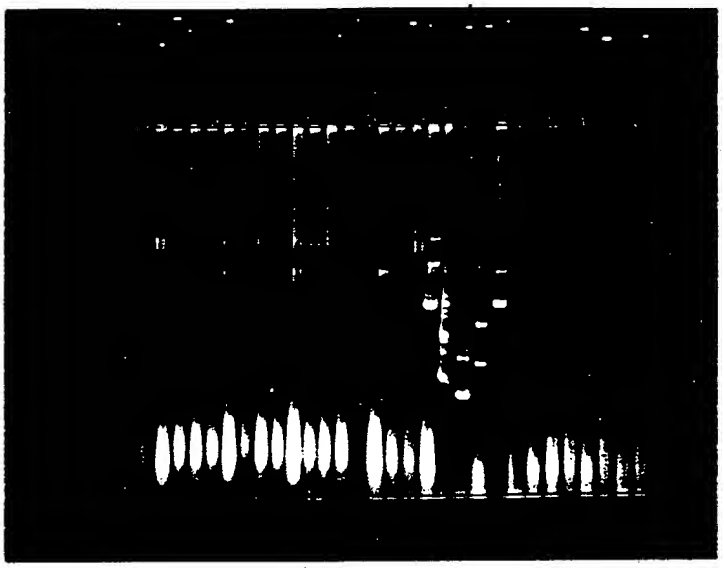
Thomas Zweg [redacted]

E2 & E3 - more pCGN cloning

resolve mini-preps of colonies on 0.5% gel

no plasmid! (with one exception!)

#19 is coincidentally the only isolate which grew significantly upon patching to G20 (others are reverting background)



cut 1 µl of 19 DNA
- 0.5 µl rox @ multi
- 0.25 µl SmaI
- 1 µl #19
- 3.25 µl dH_2O

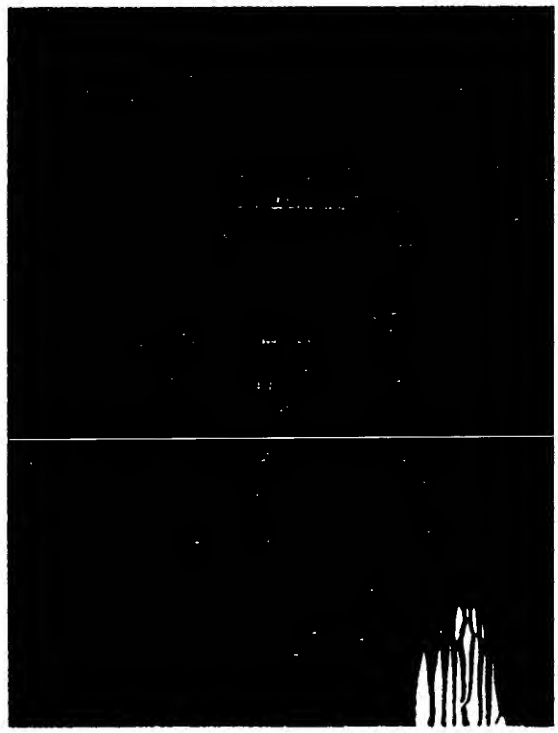
migration distance	mouse	clone
4.3kb	3.6	3.1
7.0kb	3.2	2.7
17.5kb	2.4	1.9
2.0kb	—	3.3

see reverse for graph ...

Result:

LiP - 2.0, 2.4, 2.9, 3.2, 7.0
total = 17.5 kb

#19 - 1.4, 2.9, 3.0, 3.2, 7.0
total = 17.5 kb



Q: is pCGN1578 map correct?

do more digests, but first,

transform comp LBA4404 w/ 5 µl expell 2½ hr. @ 28°C
plate on YEP 20G @ 28°C

Thomas Zuglaff

[redacted]

More E2 & E3 in pCGN1578 ...

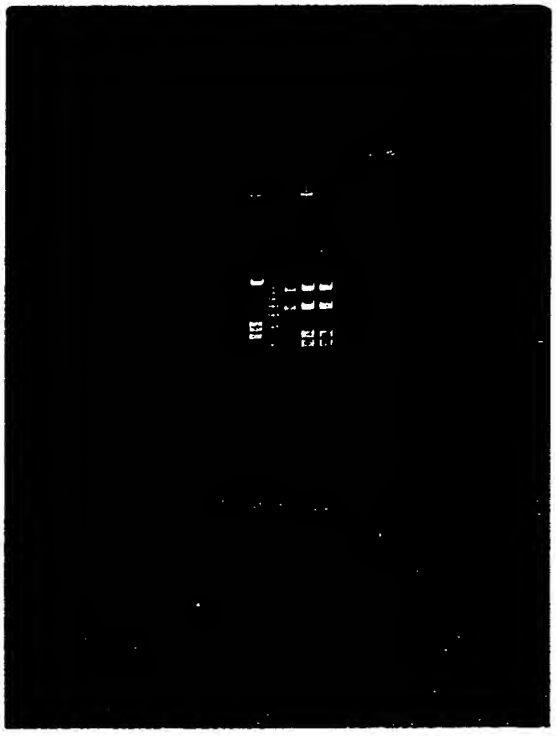
digest 318-5 with SmaI

digest 260-6, 318-5, #19 with EcoRI (multi)

all should have 1.0, 4.6, 7.3

260, 318 should also have ~2.4 kb fragment, ~2.0 kb
E2 " " " ~0.95, 1.45, ~2.0 kb

Result: PERFECT!



318-5 - fragments of
~2.4, 2.9, 5.2, 9.5 (180 mA)
(with SmaI)

others are as predicted

CONCLUSION:

- map of pCGN1578 is missing a SmaI site @ ~1600-1750
- E2 construct (#19) is <u>correct</u>!

THEREFORE,

- bona fide gent^R transformants are recovered at very low frequency
- reversion frequency (spontaneous DH5α gent^R) is significant (~ 10^{-6}-10^{-7}) but these are much weaker growers than gent^R transformants

X [redacted]

X _____

E2 & E3 - pCGN constructs

using ligation mixtures described on p. 20, retransform comp DH5α and plate <u>everything</u> ...

- E2 1 ml on 3 plates
- E3 2 ml on 6 plates

Result: E2 15 lg. colonies
E3 5 " " (3 lg., 2 med., actually

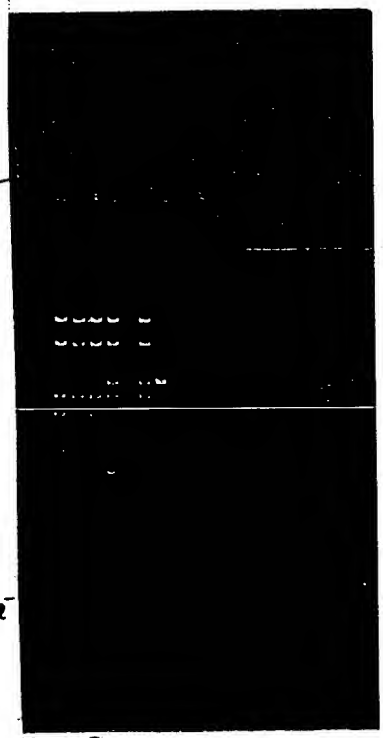
Note: control transformation with 260-6 shows that lg. colonies correspond to <u>true</u> transformants (efficiency of transf. with 260-6 was comparable to $p^{MD4.21}$, a pBR322 derivative)

start 1.5 ml LB 20G cultures for mini-prep (3 E2 clones, 6 E3 clones)

harvest after ~8 hr growth @ 37°C
E2-1-3, E3-1-3 are good E3-5,6 are weak and E3-4 is dead

3/24 - mini-prep on cell pellets

digest with EcoRI in "H" buffer



all E2 isolates look good
all E3 " " <u>bad</u>

- poor growers (on 20G) #5,6 appear to have no plasmid (as expected from previous results)
- #1,2 are indistinguishable from original 260-6 clone and probably represent background of single cut vector

Thomas J. [redacted]

pCGN-E3, the continuing saga...

Since no "real" E3 transformants were recovered, try again

- use vector purified as previously (p20)
- use insert " " (p15)

Ligation:	old	new
vector	4	6.5
insert	2	2
10x buffer	1	1
T4 ligase	0.5	0.5
dH_2O	2.5	—

transformants:

test by EcoRI digestion of minilysate DNA

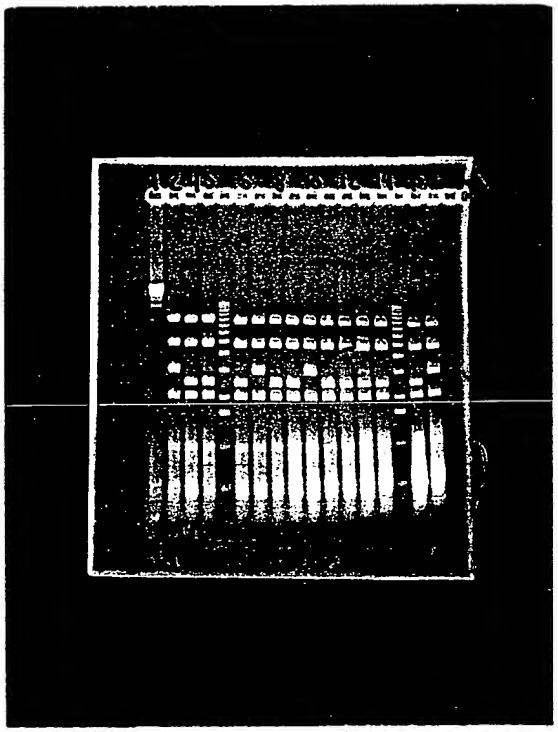
#s 2, 7, 10, 16 are correct!
with the exception of #1 (?!), all others are 260-6 recovered

transform #2 into comp Dgero (2µl DNA + 100µl cells)

approx 500 transformants obtained (YEP 20G)

start culture for plant transformation

Thomas Zügel



E2 - Western blot (std. dilutions, callus, leaves)

conc. of E2 is 1 mg/ml
- dilute 1:100 in 0.1 mg/ml B/t
- load 1, 2, 5, 10 µl of diluted enzyme (10, 20, 50, 100 ng)

grind tissue (Tobacco transformation) in 1x sample

use 1:50 dilution of anti E2 #6 (Diana Irwin's recommendation)

very good signal @ 10 ng E2

- approximately 20 µl of tissue (20 mg)
- assuming ~1% as soluble protein = 200 ug total
- load ~ 1/4 th of total (= 50 ug)
- signal looks like ~ 3-10 ng (i.e. - a high % of total!)

VERY PROMISING! (but is it active?...)

* plant-expressed E2 is ~ same MW as purified enzyme

E2 Western blot

as on p27, dilute E2 stock in dH_2O/BSA 0.1 mg/ml

1, 2, 5, 10 µl of 1:100 dilution (10–100 ng)

test selected extracts from p29

Result: 6/3

lane			
1	MW marker	10% gel	
2	10 µl E2 d.l.		
3	5 " "		
4	2 " "		
5	1 " "		
6	CT 1	1 µl each	none detect.
7	" 2		none detect.
8	" 8		< 1 ng
9	" 10		≪ 1 ng
10	" 13		none detect.
11	" 25		~ 2 ng
12	" 30		~ 3 ng
13	" 38		~ 2 ng
14	" 39		none detect.
15	" 30	~~(1:10 dilution in dH_2O + BSA)~~ 0.1 mg/ml 0.2 µl	< 1 ng

Result: Western blot agrees (for the most part) with activity measurements, with some notable exceptions:

① based on CMC, CT13 should have yielded protein
② based on MUCB, CMC CT39 " " " "
③ CT25, 38 show good protein yield, despite low MUCB activity

- based on MUCB assay, expected ~ 500 ng E2
- observed less than 10 ng, therefore activity is a very poor measure of expression
- only about 0.1% total extracted protein is E2

Thomas Zeglhofer [redacted]

Update on plant analysis - W38/MAC-E2 (TZA1)

Analysis to date: ① reducing sugar assay using CMC as substrate, ② MUCB fluorometric assay substrate ③ Western blot using anti-E2 antiserum.

Results: ① based on Western blotting, E2 expression is @ ~ 0.1% total extracted protein for CT25, CT30, CT38 and is barely detectable in CT8, CT10. Other plants tested "contained undetectable amounts of E2". ② Although there is some correlation between CMC or MUCB activity and the presence of E2 (as determined by Western), the values obtained are orders of magnitude higher than would be expected, suggesting synergistic activity with endogenous cellulase activities. ③ Using pure E2 provided by D. Irwin, approximate K_m, V_{max} for MUCB are ~9 μM and ~0.12 mole/min mole, respectively.

Conclusions: A better assay method is needed. ① CMC/reducing sugar assay is probably not practical for lg. #'s of samples ② MUCB assay could be improved by (A) extracting juice directly (no buffer) followed by (B) heating @ 60°C for 30 minutes. Lower conc. of substrate (c) in the 20-100 μM range and the addition of 1 mM gluconolactone to inhibit β-glucosidase activities may also help. Could try filter adsorption of heat-treated sample to further clean up (Millipore Ultrafree-DEAE$_{MC}$). ③ For the time being, screen plants by Western blot until assay is improved.

Thomas Ziegelhoffer [redacted]

E2 Western blot (W38/MAC$_p$-E2 TZA1)

grind ~ 20 mg leaf tissue / in 40 µl buffer (0.1% SDS also) (50mM NaOAc pH5.5, 100 mM KCl). load 2 µl of extract / lane. save remainder for juice extraction / MUCB assay (freeze leaf t. @ -80°C)

1	(control)	
2	1 µl of E2 dilution (10 ng)	
3	CT6	
4	CT9	
5	CT11	
✓ 6	CT15	
7	CT16	
✓ 8	CT17	
✓ 9	CT18	
10	CT20	✡
11	CT22	
✓ 12	CT26	
13	CT36	✡
14	CT40	✡
15	CT84	

1	CT85	✡
2	CT86	✡
3	CT87	✡
4	1 µl of E2 dilution	
5	CT88	✡
6	CT89	
7	CT90	✡
✓ 8	CT91	
9	CT92	
10	CT94	✡
11	CT95	
12	CT96	✡
✓ 13	CT97	
14	CT99	✡
15	CT100	✡

10% gel → transfer → Western (anti E2 solution, used 2x prev.)

RESULT: Those marked with a check show little or no E2 protein. ✡ indicates doublet (modification?) Extracts are ~ 1 mg/ml. Estimate that CT96, CT99 are ~ 2 ng phytase in 2 µg total protein.

Thomas [redacted]

E3 western blot (tobacco transformants)

Grind ~40 mg leaf tissue in 80 µl "buffer 1" (see p. 44), spin down pellets, recover sup (done by BK on 7/8/96).

prepare samples: 20 µl extract + 5 µl 5x buffer

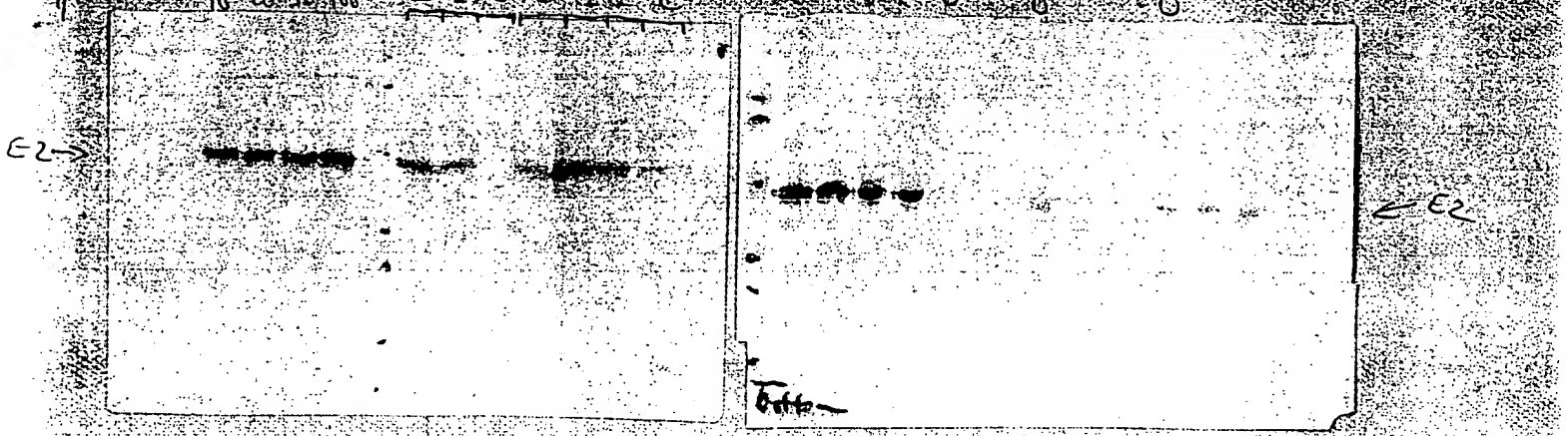
Lane #	gel 1	E3?	gel 2	E3?	E3 diluted into dH2O + 0.1% bsa
1	MW marker →			↓	
2	10 µl E3 1:100 100 ng		8 µl CT60 sample	–	
3	5 µl " " 50 ng		61	++	10% gels
4	2 µl " " 20 ng		63	+/–	
5	1 µl " " 10 ng		64	–	
6	8 µl CT47 sample	? (–)	66	–	1:200 of
7	" 48	+	68	+	E3 antiserum
8	49	+	69	–	
9	51	+	71	+/–	
10	52	+/–	72	+	
11	53	+/–	75	+++	
12	54	++	76	+/–	
13	55	++	77	+/–	
14	57	++	[78]	"lost" (tube leak)	
15	59	–	79	++	

Result: all are significantly less than 10 ng control
CT75 appears to be highest (maybe ≤ 5 ng).
A cross-reacting band @ ~ 30 kDa was present in all lanes.

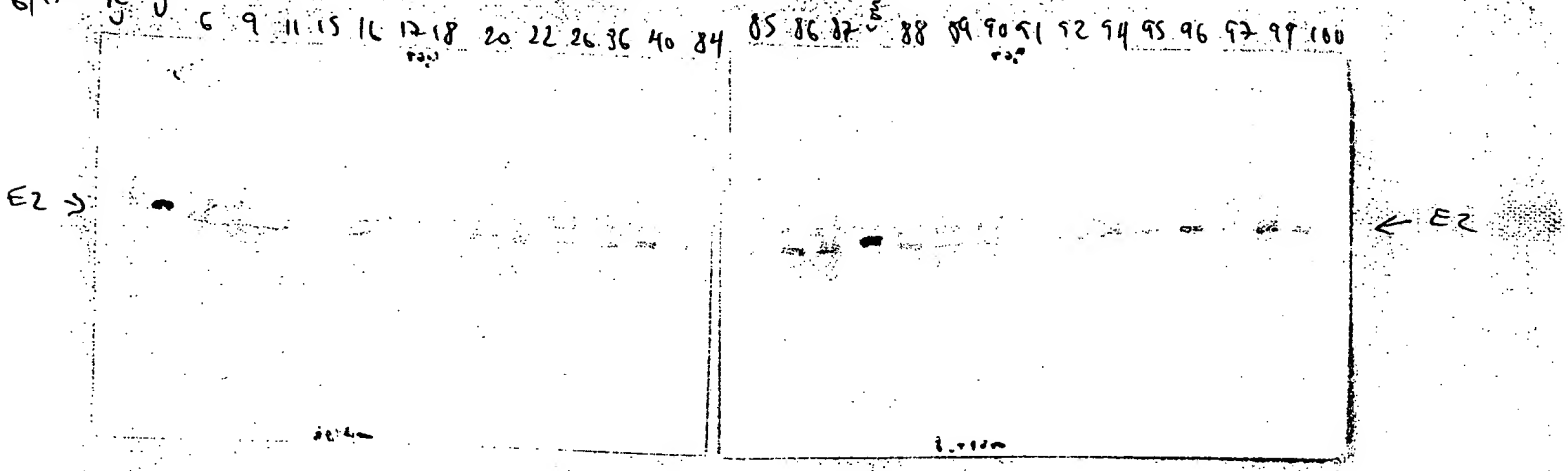
Thomas J. Kelly

[redacted]

4/18



6/17



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